IDENTIFICATION AND CHARACTERIZATION OF MAJOR CAUSATIVE BACTERIAL AGENTS OF DIARRHEA FROM REGIONS OF HIMACHAL PRADESH.

SYNOPSIS

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ABSTRACT

Diarrheal disease is responsible for 0.5 million children (≤5 years) deaths/annum globally and India accounts for approximately 0.3 million deaths. Present study is first of its kind from Himachal Pradesh to investigate role of pathogenic bacteria; diarrheagenic *E. coli* (DEC), *Vibrio spp., Salmonella spp.* and *Shigella spp.* in diarrhea incidences.

Five hundred seventy two stool specimens from diarrhea patients were screened for various bacterial pathogens through biochemical and molecular methods. Owing to higher incidences of DEC and *Vibrio spp*. in present study isolates were further characterized through molecular methods and their associations with antibiotic resistance were also correlated. DEC were identified as distinct molecular pathotypes by amplification of virulence genes (*eae, bfpA, estA, eltB* and *pCVD*) associated with pathotypes followed by sequencing. Correlations between DEC molecular pathotypes and serogroups were also studied by somatic 'O' antigen serotyping. Similarly, *Vibrio* isolates were also characterized through 16S rRNA gene amplification. Antibiotic susceptibility test and minimum inhibitory concentration were also determined for commonly used antibiotic regimen.

Biochemical characterization elucidated diarrheagenic *E. coli* as the predominant pathogen (22.7%) followed by *Vibrio spp.* (5%), *Salmonella spp.* (0.8%) and *Shigella spp.* (0.3%) among all diarrheal age groups. Our observations showed higher incidences of DEC and *Vibrio spp.* among patients so these bacterial species were taken further for characterization. DEC pathotypes were identified among 21% population. DEC infection rate was higher in children as compared to other age groups. Enteropathogenic *E. coli* (EPEC), a molecular pathotype of DEC was found as a predominant pathotype with highest frequency of 13.7%. Two other molecular pathotypes Enterotoxigenic *E. coli* (ETEC) and Enteroaggregative *E. coli* (EAEC) accounted for 5.7% and 1.3% respectively for all diarrhea incidences. Serological analysis deciphered somatic antigens O26, O2 and O3 as the major serogroups identified among EPEC, ETEC and EAEC pathotypes, respectively. DEC pathotypes exhibited high levels of resistance against all tested antibiotics, while low resistance profile for co-trimoxazole & norfloxacin reinforcing their continuance as potent antibiotics against DEC pathotypes.

DEC pathotypes significantly contribute to diarrhea morbidity and mortality in the region and elucidation of studied DEC pathotype associated clinical symptoms and resistance patterns could aid in management of diarrheal disease.

INTRODUCTION

Diarrheal diseases are major cause of morbidity and mortality in low to middle income countries and estimated to be second leading cause of mortality among children less than five years of age, resulting in 0.5 million deaths globally (WHO, 2013). Sub-Saharan & South East Asian regions account for highest burden of the disease (>72%) (UNICEF, 2017). Unfortunately, India bears highest toll of the disease which demands acceleration in interventions for diarrhea prevention and cure. Despite the well known fact that diarrheal diseases are transmitted by fecal oral route (Amézquita-Montes *et al.,* 2015) inexorable outbreaks continue to be a scourge globally (Lee *et al* ., 2016). Infectious milieu of diarrhea shed light on its multifactorial nature and vast array of disease etiology (Kotloff *et al.,* 2012). Among bacterial pathogen DEC, *Vibrio spp*., *Salmonella spp.* and *Shigella spp.* are observed as chief etiological agents. Virulence arsenal of etiological agents varies with geographical features and become significantly evident in choice of treatment protocol. Therefore, for a successful treatment regime, identification of etiological agents is of utmost significance.

Diarrheagenic *E. coli* (DEC) signifies as an important etiological agent of moderate to severe diarrhea. *Escherichia coli* are enormously versatile bacterium which elaborates its commensal and pathogenic potential in human host. Diarrheagenic *E. coli* (DEC) is reported as one of the leading causes of gastrointestinal disorders worldwide and signified as an important issue to address in public health (Frank *et al.,* 2011; Radosavljevic *et al.,* 2016; Guzman-Hernandez *et al.,* 2016; Ochi *et al.,* 2016). In low to middle income countries, >40% of diarrheal episodes among children are caused by diarrheagenic *E. coli* pathotypes (Miliwebsky *et al.,* 2016). These pathotypes also play a considerable role in diarrhea morbidity in the Indian population (Shetty *et al.,* 2013; Dutta *et al.,* 2013; Suganya *et al.,* 2016). Remarkably, distinct DEC pathotypes display specific virulence arsenal which transforms the predominant repertoire available for diagnostic and therapeutic approaches. DEC is further catalogued into various pathotypes based upon occurrence of these unique virulence determinants contributing to specific pathophysiology (Nataro & Kaper., 1998) viz. Enteropathogenic *E. coli* (*eae, bfpA*), Enterotoxigenic *E. coli* (*eltB* and *estA*), Enteroaggregative *E. coli* (*pCVD*), Enterohemorrhagic *E. coli* (*vt1* and *vt2*) and Enteroinvasive *E. coli* (*ial*) (Nataro & Kaper., 1998; Kaper *et al.,* 2004; Feng *et al.,* 2014) .

Enteropathogenic *E. coli* (EPEC) is frequently associated with diarrhea incidences from both community and healthcare settings. EPEC has been categorized into atypical and typical EPEC by the presence of *eae* gene alone and simultaneous expression of *bfpA* & *eae* genes, respectively (Nataro & Kaper., 1998; Kaper *et al.,* 2004).

Enterotoxigenic *E. coli* (ETEC), another *E. coli* have been reported as a significant pathogenic form responsible for diarrhea in travelers and population inhabiting endemic regions globally (Dutta *et al.,* 2012; Nguyen *et al.,* 2005; Panchalingam *et al.,* 2012). ETEC in the stool specimen may be confirmed by amplification of two marker genes *estA* and *eltB,* which encode heat stable and heat labile secretory enterotoxins, respectively.

Since the last decade, several reports have been published for identification of adherent Enteroaggregative *E. coli* (EAEC) as an emerging enteropathogen responsible for adult and childhood diarrhea worldwide (Weintraub *et al.,* 2007; Okhuysen *et al.,* 2010; Jensen *et al.,* 2014; Elias *et al.,* 2016). The plasmid encoded gene probe *pCVD* which elucidate aggregative phenotype is utilized for identification of EAEC in diagnostic and epidemiological studies (Nataro & Kaper., 1998; Panchalingam *et al.,* 2012; Debroy *et al.,* 1994).

Another molecular pathotypes of *E. coli*; Enterohemorrhagic *E. coli* (EHEC), a subgroup of shiga toxin producing *E. coli* and Enteroinvasive *E. coli* (EIEC) cause a devastating form of gastrointestinal infections which may lead to severe life threatening complications like haemolytic uremic syndrome (HUS). EHEC colonizes large intestine and secretes toxins (Nataro & Kaper., 1998). EIEC invades small bowel enterocytes and is regarded a true intracellular pathogens. However both EIEC and EHEC display low levels of incidences (Rasko *et al.,* 2011; Frank *et al.,* 2011).

E. coli serogrouping is used as a conventional method for pathogen characterization and diagnosis (Nataro & Kaper., 1998; Kaper *et al.,* 2004). Besides, potential use of O antigen characterization, DEC associations with O antigens vary across different geographical regions (Campos *et al.,* 2004; Tahden *et al.,* 2011). Since DEC pathotypes possess a large number of different 'O' somatic antigen, therefore their continuous monitoring is helpful in subtyping of strains and enhancing phylogenetic studies.

Vibrio, member of family *Vibrionaceae* is a comma shaped, gram negative, aquatic bacterium which colonizes small intestine of human (Kaper *et al.,* 1998). Infections caused by *Vibrio* are typically categorized into *Vibrio cholera* and non-cholera *Vibrio* infections. *Vibrio spp.* causes

profuse watery diarrhea and is historically known for worldwide epidemics and pandemics (Nair *et al.,* 1994). Symptoms of *Vibrio* mediated gastroenteritis include rice watery stools, severe dehydration, fever and vomit. *Vibrio* species expresses several virulence factors like toxins (*ctxA*, *stn*, *OmpW* and *toxR*), colonization factors (lipopolysaccharides, flagellar components, outer membrane proteins, hemagglutinins, *tcpA*), protease (hemolysins, cytolysins, thermolysins; metalloproteases) and iron acquisition systems (Almagro-Moreno *et al.,* 2015). Cholera toxin encoded by *ctxA* gene has been isolated only from clinical strains and is utilized as an epidemiological marker to access toxigenic potential of *Vibrio* strains (Jiang *et al.,* 2003; Li *et al.,* 2015).

Since diarrheal disease is generally self-limiting, antidiarrheal agents are not usually recommended for treatment of diarrhea. However, traveler's diarrhea, persistant diarrhea and acute invasive diarrhea display high severity of infection and extended recovery periods which reinforce the use of antimicrobials like ampicillin, norfloxacin, nalidixic acid, cefixime and cotrimoxazole (ICMR SOP, 2015)

Diarrhea disease is one of leading cause of morbidity and mortality in developing nations. Diarrhea associated high rates of hospitalization and outpatient visits reinforce information on infection etiology, its determinants, preventive and control interventions for better planning and organization of health services. Comprehensive studies investigating the role of DEC pathotypes are scarce in India and are mainly restricted to South Indian population. However studies analyzing epidemiological significance of DEC are not reported till date from Himachal Pradesh. Present study focuses on investigating the prevalence of diarrheagenic *E. coli* pathotypes with exhibited serogroups in Himachal Pradesh, a northern hilly state of India. Molecular methods were utilized to better define DEC incidences, their etiology and clinical outcomes. Correlation of DEC pathotypes with different age groups and clinical symptoms were also analyzed. The study also encompasses resistance patterns of identified *E. coli* pathotypes and *Vibrio spp*. which will be useful in treatment regimes for tackling these specific pathogens.

OBJECTIVES

1. To collect diarrheal samples from different regions of Himachal Pradesh and to correlate epidemiological details and medical history of patients.
2. To isolate different bacterial pathogens from diarrheal samples and identification using standard microbiological and biochemical methodologies.
3. To characterize the different pathotypes of diarrheagenic *E. coli* and *Vibrio spp.* using molecular and serological methods and establishment of these pathogens prevalence in Himachal Pradesh.
4. To estimate the level of resistance of DEC and *Vibrio spp.* against different antibiotics (commonly used in diarrheal therapies) using MIC determination and antibiogram formation.

Methodology:

2.1 Collection of diarrheal samples and study subjects

From February 2013 to April 2016, a total of 572 stool specimens of diarrheal patients aged between 13 days to 85 years were collected. Samples were collected from patients with primary complaint of three or more loose stools/day who were admitted to Regional hospital, Solan and tertiary care hospital Indira Gandhi medical college (IGMC) Shimla Information on gender, age, geographic origin and clinical symptoms was obtained by means of standard questionnaire. Patients presented with loose stool as chief complication but also reported to have other clinical manifestations like dehydration, vomit, fever, abdominal pain, mucus, in common. Written informed consents were taken from patients or patient's parent or legal guardians in case of children.

Stool specimens from diarrheal patients were collected in sterile containers and transported immediately to the laboratory after collection. All experiments included in the study were authorized by the Institutional Ethics Committee (IEC/Project no-04-2014).

2.2 Microbiological and biochemical characterization of bacteria

The stool specimens were enriched in Luria Broth and alkaline peptone water and streaked onto MacConkey agar, eosin methylene blue agar, thiobacillus citrate bile salt agar (TCBS), xylose lysine deoxycholate (XLD) agar and incubated for 24 to 48 hours at 37°C. For isolation of DEC, typical lactose fermenting pink colored colonies from MacConkey agar were selected and sub-cultured on Luria Bertani agar. For *Vibrio spp.,* golden yellow colonies from TCBS agar were picked and inoculated into alkaline peptone. Red and red colonies with black centres were

chosen from XLD media for *Shigella spp.* and *Salmonella spp*. respectively. Following overnight incubation, selected colonies were subjected to a series of standard biochemical tests; IMViC (indole, methyl red, Voges Prausker, citrate), triple sugar iron agar, urease agar and motility tests (Panchalingam *et al*., 2012). Bacterial strains with characteristic IMViC pattern were biochemically characterized as *E. coli, Vibrio spp., Shigella spp., Salmonella spp.* respectively**.**

2.3 DNA extraction & 16S rRNA gene characterization

Biochemically confirmed strains were initially molecularly characterized using 16S rRNA gene for *E. coli* and *Vibrio spp.* DNA extraction of *E. coli* strains was performed by phenol chloroform method as described previously by Sabat *et al.,* 2000. PCR thermo cycling conditions for 16S rRNA gene were standardized at following condition. Initial denaturation was done at 94°C for 5 min, final denaturation 94°C for 30s, primer annealing at 52°C for 30s, and initial extension at 72°C for 30s for 35 cycles, with a final extension of 7 minutes at 72°C. PCR reaction was set up with 25µl reaction mixture having 12.5 µl 2X PCR Master Mix, 0.2 µM of each primer, 300 ng/µl of template DNA and nuclease free water.

The molecular characterization of *Vibrio spp.* was performed by PCR amplification of 16S rRNA gene. Primers used for molecular identification of *Vibrio* species included forward primer 5'-CGGTGAAATGCGTAGAGAT -3' and reverse primer 5'-TTACTAGCGATTCCGAGTTC-3' (Lipp *et al* . 2003). 2µl of DNA extracted by colony heat boiling was amplified in a 15µl PCR reaction mixture; 7.5µl of 2X PCR master mix, 0.25µl of each primer and nuclease free water.

PCR amplification was accomplished using following conditions: 1 cycle of pre-denaturation at 95˚C for 5 minutes followed by 35 cycles of final denaturation at 95˚C for 30 seconds, annealing at 48˚C for 40 seconds, extension at 72˚C for 50 seconds, and final extension at 72˚C for 7 minutes. A no template PCR was run as negative control for the reaction. The amplified PCR products were analyzed with ethidium bromide stained 1.5% agarose gel (in 1X TAE buffer) and visualized using UV trans-illuminator.

2.4 Molecular characterization of DEC pathotypes

DEC molecular pathotypes were identified by amplification of virulent genes. Primer sequences were selected from two published studies (Nguyen *et al.,* 2005, Panchalingam *et al.,* 2012). Initially, molecular pathotypes were amplified in a multiplex PCR followed by single gene PCR for identification and reproducibility of specific DEC pathotypes. Different molecular pathotypes were identified on the basis of amplification of following amplicons of genes; ETEC encoded

heat stable (*estA* 147bp) and heat labile toxin (*eltB* of 322bp or 508 bp) genes, EPEC encoded bundle pilus forming gene (*bfpA* 367bp) and intimin gene (*eae* of 830 or 376 bp amplicon), EHEC encoded verocytotoxins (*vt1* 130bp and *vt2* 298bp), EIEC encoded invasion gene (*ial* 320bp) and EAEC plasmid encoded aggregative phenotype specific (*pCVD* 630bp) were targeted in the PCR. During EPEC pathotype identification, *eae* and *bfpA* genes amplify at 367bp and 376bp respectively (Nguyen *et al.*, 2005), which could not be resolved by agarose gel electrophoresis, therefore, *eae* gene (830bp) and *bfpA* gene (367bp) were stringently amplified by single gene PCR. PCR thermocycling conditions for pathotypes were same as described above for amplification of 16S rRNA gene. Amplified PCR products were further confirmed by commercial Sanger sequencing at various time intervals during study. Sequenced DEC pathotypes were taken as positive control in PCR reaction.

2.5 Serological characterization of DEC molecular pathotypes

Identification of bacterial somatic O antigen was performed by standard agglutination test using 176 'O' specific antisera (Ørskov & Ørskov, 1984). For serogroups characterization, biochemically and molecularly confirmed *E. coli* isolates were screened at National *Salmonella* and *E. coli* centre at Central Research Institute, Kasauli (H.P.). Briefly, test strain was inoculated into 5ml nutrient broth and incubated at 37°C for overnight with agitation. Bacterial growth was boiled at 100°C for one hour and then formalin was added to a final concentration of 0.3% (Test antigen). For testing with pooled sera, 50µl of 16 pools of O antisera were added to 96 well plate. Then 50µl of test antigen was added to each well. A negative control well was set up with 50µl each of antigen and saline respectively. Plates were incubated at 37°C overnight and observed for agglutination reaction. Test strain showing agglutination in all wells including negative control, strain was regarded 'rough'. If agglutination was seen with single pool, then next agglutination test was setup with factor sera constituting the pool. But if agglutination was seen with more than one pool, then antigen was titrated against all sera constituting the pools. The test antigen which even didn't show agglutination following antigen preparation at 121°C for 2½ hours was regarded as 'untypeable'.

2.6 Antimicrobial susceptibility test (AST) and minimum inhibitory concentration determination (MIC)

The antimicrobial susceptibility of the PCR positive *E. coli* pathotypes was determined by standard Kirby Bauer's disk diffusion method (Bauer *et al* ., 1959) against ampicillin (10µg),

cefixime (5µg), co-trimoxazole (25µg), nalidixic acid (30µg) and norfloxacin (10µg) according to CLSI and ICMR guidelines (Patel *et al.*, 2015; ICMR SOP, 2015). Minimum inhibitory concentrations for ampicillin (0.016-256 µg), cefixime (0.016-256 µg), co-trimoxazole (0.016-256 µg), nalidixic acid (0.016-256 µg) and norfloxacin (0.016-256 µg) were determined using the E-test. *Escherichia coli* ATCC 25922 was used as reference strains for quality control in AST & MIC tests. Results were interpreted according to CLSI and ICMR guidelines (Patel *et al.*, 2015; ICMR SOP, 2015).

Similarly, for *Vibrio spp.* Kirby Bauer's disk diffusion on Mueller Hinton agar was used to evaluate antibiogram patterns against fifteen antibiotics; cefepime (30µg), ceftazidime (30µg), ceftriaxone (30µg), imipenem (10µg), netilmicin (30µg), amikacin (30µg), kanamycin (30µg), tobramycin (10µg), streptomycin (10µg), ofloxacin (5µg), ciprofloxacin (5µg), levofloxacin (5µg), norfloxacin (10µg), nalidixic acid (30µg) and vancomycin (30µg). Results were interpreted according to manufacturer's instruction for *Enterobacteriaceae* family.

2.7 Statistical analysis

The age of the patients were classified into five groups viz., <2 years, 3–5 years, 6–17 years, 18-65 years and >65 years. In statistical analysis >65 years age represented the most normative group because elderly from developing countries are more prone to diarrheal infection due to immunocompromised status (Zhang *et al* ., 2016). In a similar study by Dutta *et al.*, 2012, elderly age group comprising subjects >65 years of age were also taken as reference for comparative statistical analysis (Dutta et al., 2012). Similarly, categorized DEC was compared among each age group with >65 years as reference group. Fisher's exact test was performed to establish mutual relatedness among the three types of DEC pathotypes. P values of ≤ 0.05 were considered as statistically significant and calculated the odds ratio (OR) at the 95% confidence interval (CI). Chi square (bivariate analysis) was used to compare clinical symptoms in DEC positive and negative populations.

3. Results:

3.1 Sample collection and diarrheal population

During February 2013 to April 2016, a total of 572 stool specimens were collected from diarrheal patients admitted to regional (Govt. hospital Solan) and tertiary care hospital (Indira Gandhi Medical College, Shimla) in Himachal Pradesh, a hilly state of India. Hospitalized patients presented a very wide age group window ranging from 13 days to 85 years. A total of

two hundred forty seven (n=247) patients less than five years of age and three hundred twenty five patients (n=325) aged >5 years were analyzed in the present study. Standard microbiological techniques and biochemical assays showed presence of diarrheagenic *E. coli* in stool specimens of diarrhea patients.

3.1 Identification & biochemical characterization of bacteria

Biochemical characterization of bacteria screened on differential and selective media elucidated diarrheagenic *E. coli* as the predominant pathogen (22.7%) followed by *Vibrio spp.* (5%), *Salmonella spp.* (0.8%) and *Shigella spp.* (0.3%) among all diarrheal age groups. Our observations showed higher incidences of DEC and *Vibrio spp.* among patients so these bacterial species were taken further for characterization.

3.2 Incidences of DEC molecular pathotypes in study population

Identification of diarrheagenic *E. coli* pathotypes was done on the basis of biochemical, molecular and serological assays. All biochemically characterized *E. coli* were also confirmed by 16S rRNA gene amplification (Supplementary Figure 1A). Further, DEC molecular pathotypes were identified by amplification of virulence gene specific primers selected according to pathotype classification system devised by Nataro & Kaper (Figures 1B-1D). Overall, diarrheagenic *E. coli* accounted for a proportion of approximately 21% (n=120/572) in hospitalized patients. Among distinct DEC pathotypes, EPEC (n=79/572, 13.8%) was found to be predominant pathotype followed by ETEC (n=33/572, 5.8%) and EAEC (n=8/572, 1.4%). Pathotypes belonging to classes EHEC and EIEC of DEC were not found in analyzed specimens.

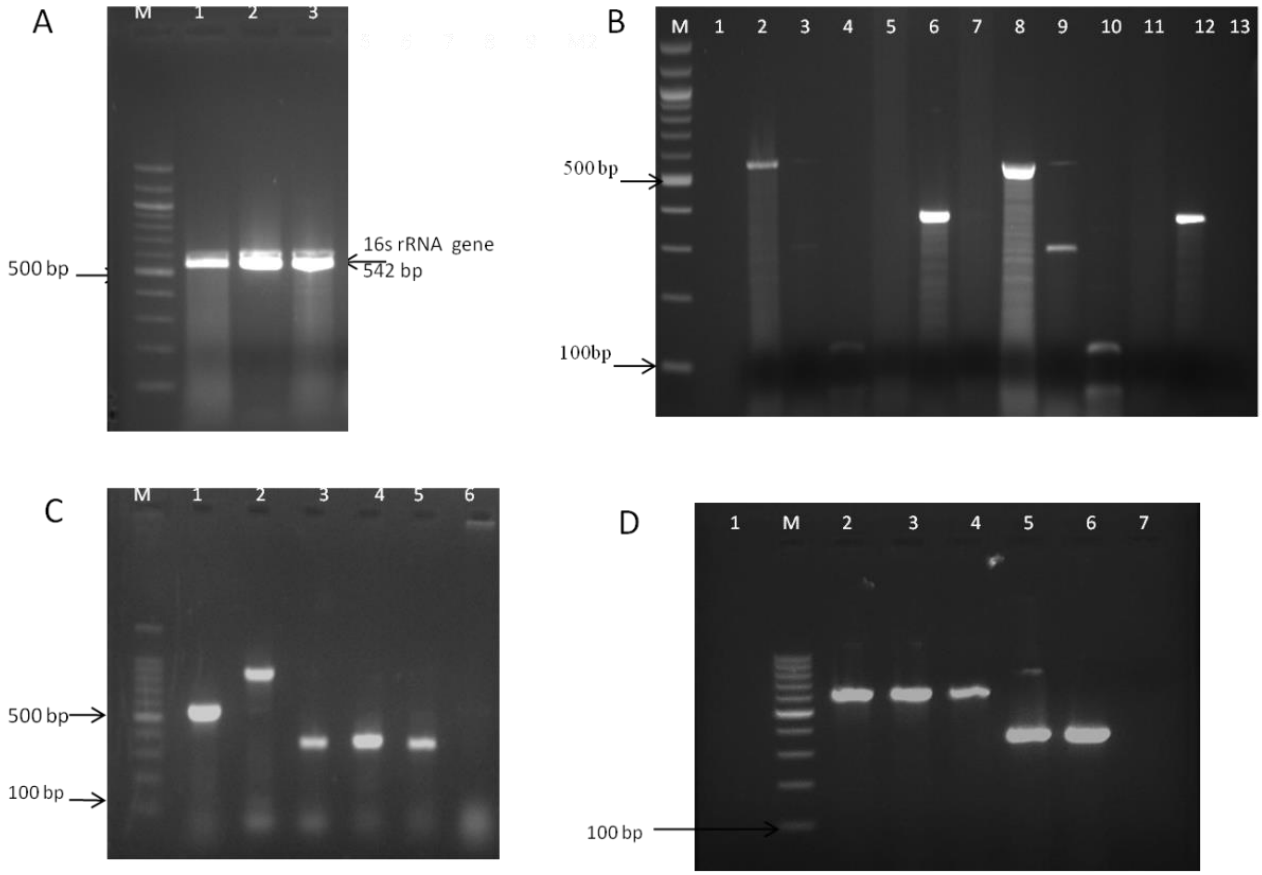


Figure 1 A. Representative PCR agarose gel (1.5% in 1 X TAE) image for genus *Escherichia* 16S rRNA gene (542 bp). Lane M represents 100 bp DNA marker (NEB). Lanes 1, 2, 3 show amplified 16S rRNA gene (542 bp), confirming *E. coli* isolates.

Figure 1 B. Represntative PCR agarose gel (1.5% in 1 X TAE) of diarrheagenic *E. coli* pathotypes. Lane M reprsents 100 bp marker (NEB) , lane1 shows negative control, lane 2 & 8 reprsent 16S rRNA gene (542 bp), lane no 3,4, 9, 10 represent ETEC (*estA* gene 147bp, *eltB* gene 322bp respectively). Lane no 6 & 12 represent EPEC (*eaeA* gene 376 bp). Lane no 7 & 13 showed no amplification.

Figure 1 C. Multiplex PCR gel image for diarrheagenic *E. coli* pathotypes run on 1.5% agarose gel in 1XTAE. Lane M 100 bp marker, lane 1 represent ETEC (*eltB* gene 508 bp), lane 2 represent EPEC (*eaeA* gene 830 bp), lane 3, 4, 5 represent EPEC (*bfpA* 367 bp). Lane no 6, 7 represent -ve control *E. coli* DH5-α & *E. coli* 25922

Figure 1 D. Representive PCR gel image for diarrheagenic *E. coli* pathotypes run on 1.5% agarose gel in 1XTAE. Lane 1 represent negative control, . Lane M 100 bp marker, lane 2, 3, 4

represent EAEC (*pCVD* gene 630 bp), lane 5 & 6 represent EPEC (*eae* gene 376 bp). Lane no 7 shows no amplification of targeted genes.

The amplified products of PCR were further confirmed by sequencing and partial coding sequences obtained were found to be 100% similar to targeted reference genes. The gene sequences were submitted to NCBI database (NCBI accessions: KX911251, KX911252, KX911253, KX911255) and were utilized as positive control in subsequent PCR analysis.

3.3 Distribution of virulent genomic elements among DEC molecular pathotypes

Characterization of DEC molecular pathotype was ascertained on amplification of either distinct gene or combination of genes. In the present study, *eae* gene of atypical EPEC (62.5% *eae* gene, n=75/120) was most prevalent as compared to typical EPEC (3.3% *eae* and *bfpA*, n= 4/120). In case of ETEC infected patients, strains harboring *estA* (18.3%, n=22/120) were more prevalent than strains possessing both *estA* and *eltB* genes (10%, n=12). All EAEC strains (n=8) possessed *pCVD* (6.6%, 8/120) gene.

3.4 Clinical symptoms Vs DEC molecular pathotypes

Clinical symptoms of DEC pathotypes mediated infection vary from acute to persistant diarrhea, febrile or afebrile, with or without symptoms of dehydration. Besides, loose stools as a common illness among study population, symptoms of fever, vomit, dehydration, mucus & abdominal pain were also observed. To ascertain DEC molecular pathotype specific clinical symptoms, chi square analysis was performed and P values & odd ratio (OR) at 95% confidence interval (CI) were calculated (Table 1).

Table 1 Comparative analysis of clinical features associated with DEC positive and DEC negative patients by chi square test.

Clinical symptoms Observed	DEC positive (n=120)	DEC negative (n=452)	P value (at 95% CI)
Vomiting	30 (25%)	109 (24.1%)	0.840
Fever	25 (20.8%)	62 (13.7%)	0.0536
Dehydration	25 (20.8%)	107 (23.6%)	0.5117
Watery diarrhea	20 (16.6%)	11 (2.4%)	0.0001*
Mucus	6 (5%)	4 (0.8%)	0.0022*
Abdominal pain	3 (2.5%)	6 (1.3%)	0.3589

(*) Mark shows statistically significant values

For comparison, DEC positive population (n=120) was taken as positive control and population without DEC infection (n=452) was utilized as negative control. Symptoms of watery stools, visible mucus were found statistically associated with DEC pathotype infection. Other patho-physiological features like vomiting, severe dehydration and fever were also observed with higher frequency in EPEC and ETEC pathotypes but similar cases were also observed in DEC negative population, therefore statistically insignificant. While, EAEC infection was found primarily associated with the frequent bowl movements (>6 episodes of watery stool), fever, vomiting and dehydration.

3.5 Age group distribution of DEC molecular pathotypes

For determination of high risk age groups, study population was stratified into five various age groups viz. children 0-2 years (n=202) and 3-5 years (n=45), adolescent 6-17 years (n=41), adult 18-65 years (n=257) and elderly >65 years (n=27). Our study revealed uniform abundance of EPEC & ETEC infections in all age segments however children less than five years (<2 years & 3-5 years) of age showed higher incidence rates as compared to any other age group (Figure 2). Although EAEC pathotype was detected with low frequency but enteropathogen was predominantly found in children population (5.2%, 7/572) as compared to adult diarrheal patients (0.3%, 1/572) (Figure 2).

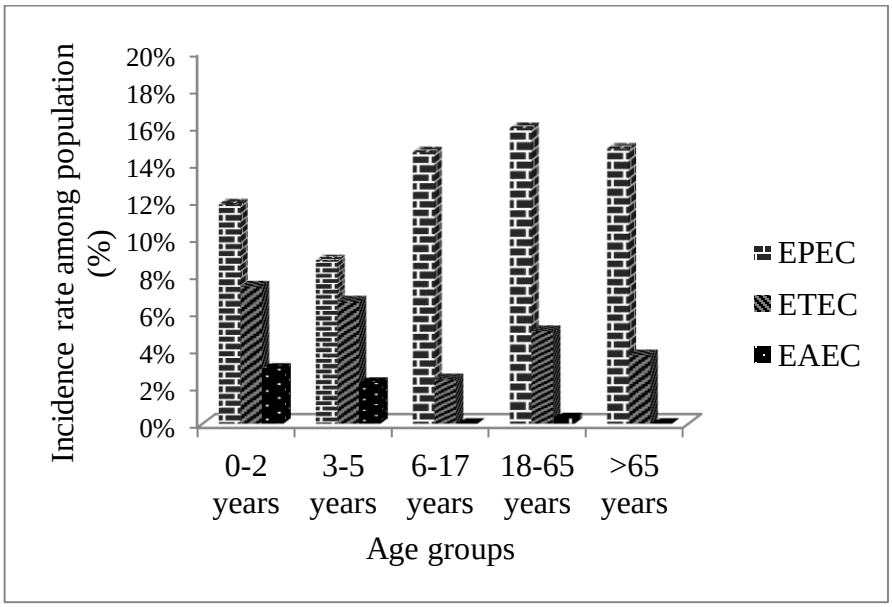


Figure 2. Prevalence of diarrheagenic *E. coli* (DEC) pathotypes among different age groups. X-Axis represents different age groups under study and Y-axis represent proportions of different

pathotypes. EPEC= Enteropathogenic *E. coli*, ETEC= Enterotoxigenic *E. coli*, EAEC= Enteroaggregative *E. coli.*

In order to recognize specificity of any DEC pathotype to particular age groups, bivariate Fisher analysis was performed (Table 2). Statistically significant correlations were observed for EPEC, ETEC & EAEC pathotypes with those of children <2 years of age. However in adult age group (17-65 years), only EPEC & ETEC prevalence was correlated significantly.

Table 2. Bivariate analysis of age wise distribution of Diarrheagenic *E. coli* pathotypes using Fisher's exact test.

Age group	DEC pathotype	Odd Ratio (at 95% CI)	p- value
0-2 years	EPEC (24/79)	8.182 (2.684-24.94)	0.0001*
	ETEC (15/33)	26.67 (3.248-219)	0.0001*
	EAEC (6/8)	44.20 (1.794-1089)	0.0070*
3-5 years	EPEC (4/79)	1.00 (0.2411-4.418)	1.0000
	ETEC (3/33)	3.1 (0.3050-31.50)	0.6312
	EAEC (1/8)	3.4 (0.1194-96.78)	1.0000
6-17 years	EPEC (6/79)	1.541 (0.4175-5.688)	0.7480
	ETEC (1/33)	1.000 (0.05988-16.70)	1.0000
17-65 Years	EPEC (41/79)	20.23 (6.743-60.69)	<0.0001*
	ETEC (13/33)	20.80 (2.522-171.5)	0.0005*
	EAEC (1/8)	3.400 (0.1194-96.78)	1.0000
	>65 years as reference category		

(*) Mark shows statistically significant values

3.6 Serogroup analysis of DEC pathotypes

Molecular pathotypes of DEC were characterized for *E. coli* somatic O antigen and were found associated with at least twenty three different O serogroups (Figure 3). Serologic analysis revealed 60.8% (73/120) of *E. coli* isolates as diarrhea associated serotypes. Serogroups O2, O26, O35 and O41 were the most commonly characterized with a prevalence of 41% (30/73).

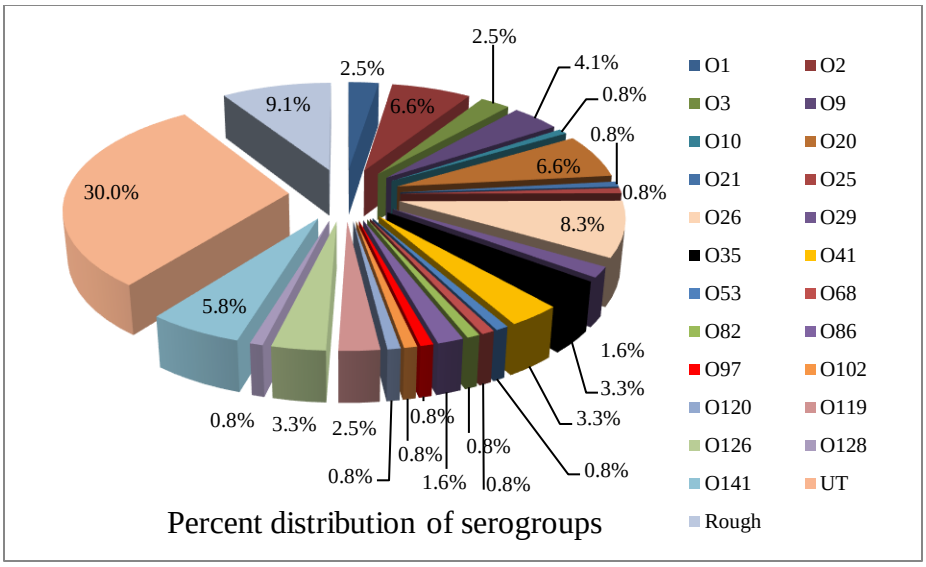


Figure 3. Distribution of different O serogroup among diarrheagenic *E. coli* (DEC) isolates. UT (untypeable), Rough (Non-agglutinable with antisera).

Figure 4 shows O26 and O2 as most commonly isolated among EPEC and ETEC pathotypes, respectively. O26 is often associated with classical attaching & effacing group (EPEC) and non O157- EHEC strains (Nataro & Kaper, 1998). We observed serogroups O2, O25 & O128 in ETEC strains only.

Apart from classical serogroups, EAEC predominantly belonged to serogroup O3 (2.5%). However, higher proportions of strains belonging to EPEC, ETEC and EAEC pathotypes remained un-typeable (30%) and didn't agglutinate with O antiserum (9%).

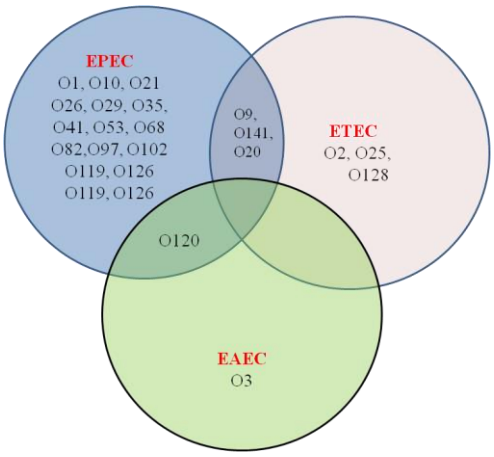


Figure 4. Interactions based upon serogroup sharing between various diarrheagenic *E. coli* pathotypes. The overlapping area is showing mutual serogroup in respective molecular pathotypes.

Correlation between DEC virulent genes and O serogroups revealed *eae* gene of EPEC was most commonly associated with more number of O serogroups (Figure 5). *estA* and *eltB* genes of ETEC toxins were observed with O2, O20, O25, O102 and O141 serogroups while serogroup O9 was observed with *estA* only. The *pCVD* gene of EAEC was found to be associated majorly with one serogroup O3.

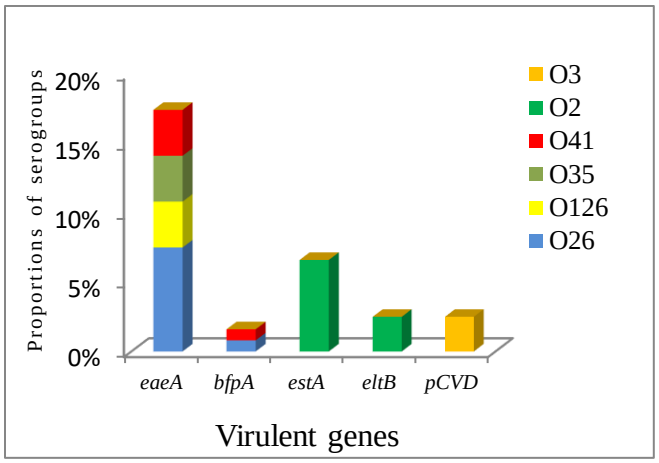


Figure 5. Representing association of diarrheagenic *E. coli* (DEC) virulent genes with top six O serogroups.

3.7 *Vibrio* incidences among population

Molecular characterization of biochemically confirmed *Vibrio* isolates showed amplification of *Vibrio* genus specific 16S rRNA gene in 5% (n= 29/572) isolates (Figure 6). Mixed enteric infection rates of *Vibrio* with *E. coli* and with rotavirus were observed to be 1.2% and 0.03% respectively. Amplified product of *Vibrio* genus specific 16S rRNA gene (663bp) was sequenced and the obtained sequence was submitted to GenBank NCBI (Accession no. KX891575).

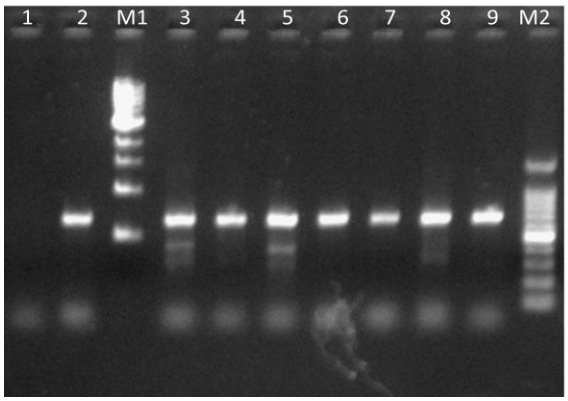


Figure 6. PCR assay for characterization of *Vibrio spp.* through amplification of 16S rRNA gene. Lane 1 shows a no template control. Lane 2 and lanes 3-9 show amplified PCR product for

Vibrio genus specific 16S rRNA gene (663bp). Lane M1 & M2 show 1Kb and 100 bp DNA ladder (NEB) respectively.

3.8 Pattern of *Vibrio* infections among different age groups

Incidences of *Vibrio* were analyzed among all five age groups i.e. infants (0-2 years), children (3-5 years), adolescents (6-17 years), adults (18-65 years) and elderly (>65 years) (Figure 7). Highest frequency of *Vibrio* infection was observed in adolescents (7.3%) and adult age (6.6%) groups, while, lowest infection rates observed among infants (2.9%) followed by elderly (3.7%) and children population (4.4%).

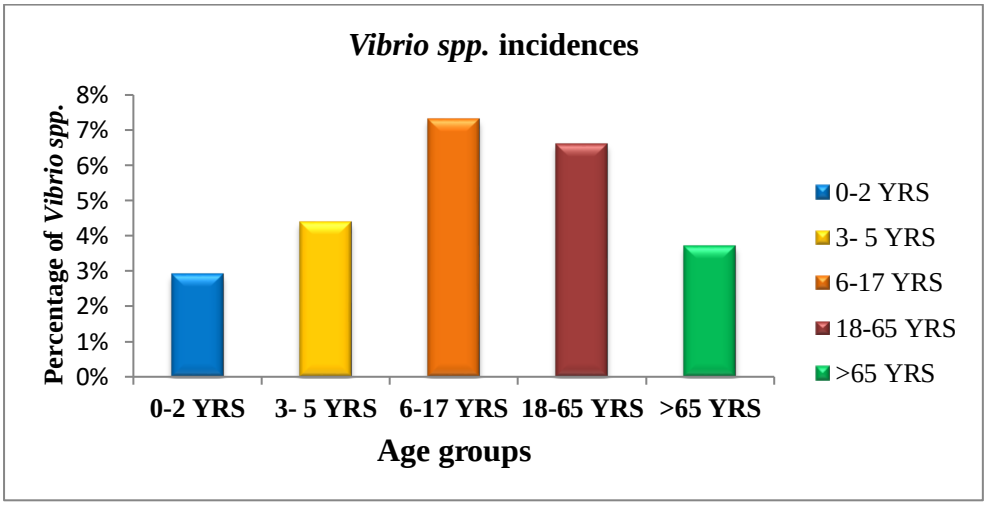


Figure 7. Incidences of *Vibrio spp.* among different age groups of moderate to severe diarrhea infected patients. 0-2 years: infant, 3-5 years: children, 6-17 years: adolescent, 18-65 years: adult, >65 years represents elderly population**.**

Fisher's exact test was calculated to decipher highest risk age group among *Vibrio* infected population (Table 3).

Table 3. Identification of high risk age group among diarrheal patients by Fisher exact test**.**

Age groups	**P –value at 95% confidence interval (CI)**	**Odd ratio (OR)**	**Significance**
0-2 yrs	0.59	0.79	NS
3-5 yrs	1.00	1.20	NS
6-17 yrs	0.92	2.05	NS
18-65 yrs	1.00	1.84	NS
>65 yrs	Reference category		

NS= Not significant

3.9 Seasonal frequency of *Vibrio* incidences

Screening of *Vibrio* species was carried out throughout the year to analyze season wise distribution (Figure 8). Season wise classification of months was performed according to Indian seasons: summer (March to May), rainy (June to September), autumn (October to November) and winter (December to February). Highest incidences of *Vibrio* were observed for rainy season (34.3%) followed by winter (27.5%) and summer seasons (23.9%). Among different age groups, infants were highly susceptible to *Vibrio* species during winter and summer seasons while, no case was reported among infants in rainy season. For adolescent and adults, being the most active and productive group highest infection was observed during rainy season followed by winter and summer. Overall, seasonality peaks observed to have a rise in summer, reached at a maximum in rainy and a final decline during autumn.

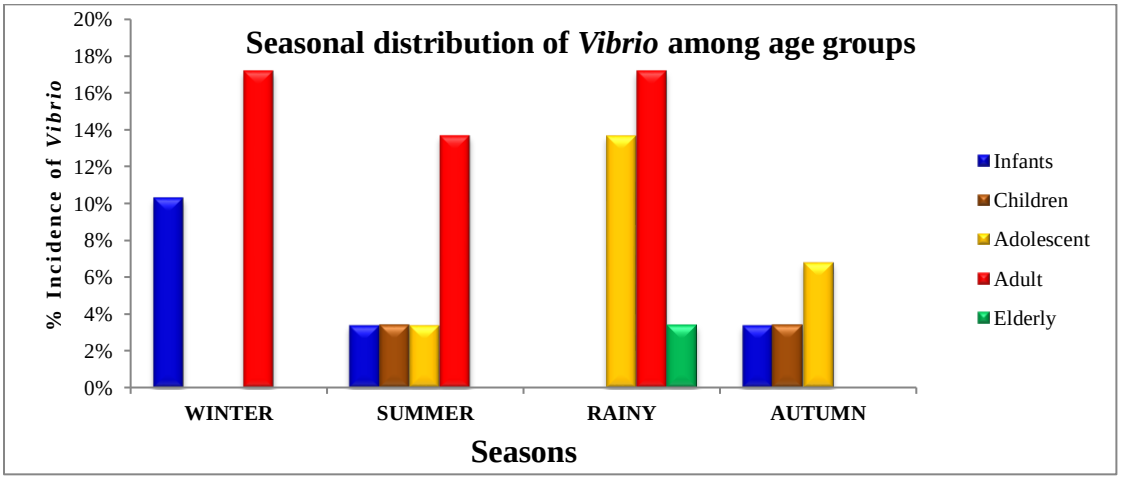
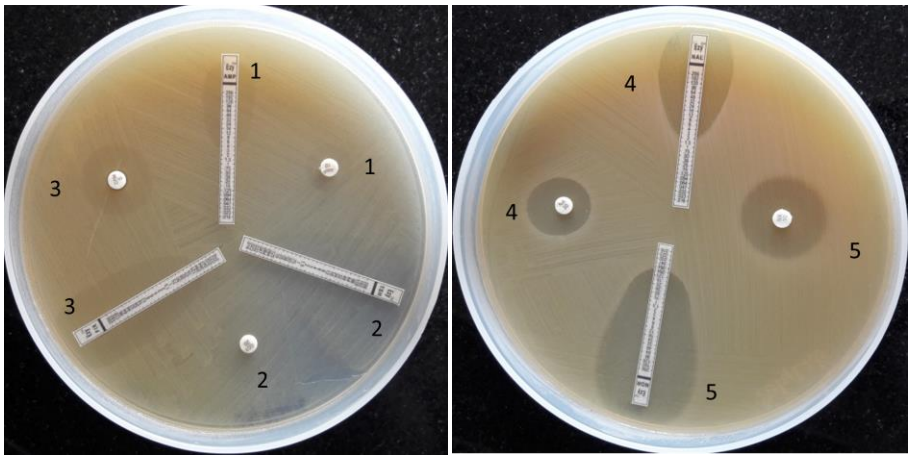


Figure 8. Seasonal trends of *Vibrio* species incidences among different age categories of diarrheal patients.

3.10 Antimicrobial resistance in DEC molecular pathotypes

Several studies have been performed for analyzing antibiotic resistance patterns among diarrheagenic *E. coli* isolates (Chen *et al* ., 2018; O'Ryan *et al* ., 2005; Dupont *et al* ., 2016; ICMR SOP, 2015). Therefore, molecular pathotypes of DEC were also screened for antibiogram patterns by antimicrobial susceptibility test (AST) and minimum inhibitory concentration (MIC) according to CLSI guidelines (Patel *et al* ., 2015). Antibiotics utilized in screening were chosen on the basis of ICMR recommendations (ICMR SOP, 2015) (Figure 9A & 9B).



A B

Figure 9. Antibiotic susceptibility test and minimum inhibitory concentration test for diarrheagenic *E. coli* pathotypes. Zones of inhibition were seen against co-trimoxazole, nalidixic acid, norfloxacin, cefixime and ampicillin in AST and MIC tests. 1 Ampicillin (10µg), 2 Co-trimoxazole (75µg), 3 Cefixime (5µg) 4 Nalidixic acid (10µg), 5 Norfloxacin (30µg).

AST and MIC revealed that a majority of diarrheagenic *E. coli* strains were sensitive for co-trimoxazole (36%), while <20% were sensitive for cefixime, norfloxacin, ampicillin and nalidixic acid (Table 4). Proportions of intermediate strains against all five antibiotic were <5%. DEC pathotypes exhibited alarming rates of resistance against widely used antimicrobials; ampicillin cefixime, nalidixic acid and norfloxacin (approximately 80%).

On correlating resistance pattern with distinct DEC pathotype, EPEC strains were observed to be most resistant against all tested drugs. ETEC strains were found 18-49% sensitive against all tested antibiotics. EAEC pathotypes showed highest sensitivity for co-trimoxazole (62.5%), norfloxacin (37.5%), nalidixic acid and cefixime (25% in both). Table 4 showed statistical analysis using chi square test performed to evaluate pathotype specific antibiotic resistance level. All molecular DEC pathotypes were found to possess significant levels of antibiotic resistance against all antibiotics (Table 4).

Table 4. Antibiotic resistance among the different diarrheagenic *E. coli* groups in patients with diarrhea. AMP=ampicillin, COT= Cotrimoxazole, CPM= cefixime, NOR=norfloxacin and NAL=nalidixic acid. * P< 0.05, ** P<0.01 (chi Square test), for the comparison of the resistance percentage among the different *E. coli*.

Antibiotics	Characteristics (Resistant)	P value	OR (95% CI)
AMP	EPEC 82.2% (65)	0.0026**	2.792 (1.456 to 5.353)
	ETEC 78.7% (25)	< 0.0001**	3.473 (1.866 to 6.462)
	EAEC 75% (6)	< 0.0001**	4.895 (2.668 to 8.979)
CPM	EPEC 77.2% (61)	0.0311*	2.052 (1.108 to 3.801)
	ETEC 81.8% (26)	0.0004**	3.329 (1.721 to 6.440)
	EAEC 75% (6)	< 0.0001**	4.895 (2.668 to 8.979)
NAL	EPEC 79.7% (67)	0.0078**	2.452 (1.299 to 4.627)
	ETEC 81.8% (27)	< 0.0001**	4.205 (2.209 to 8.005)
	EAEC 62.5% (5)	< 0.0001**	3.807 (2.078 to 6.974)
NOR	EPEC 79.7% (67)	0.0078**	2.452 (1.299 to 4.627)
	ETEC 69.6% (22)	0.0135*	2.154 (1.205 to 3.849)
	EAEC 62.5% (5)	< 0.0001**	3.807 (2.078 to 6.974)
COT REFERENCE CATEGORY			

3.11 Antimicrobial resistance patterns of *Vibrio* species

According to Indian Council of Medical Research (ICMR) different antibiotic classes; tetracycline, β–lactam and quinolones are prescribed for treatment of severe diarrhea cases. Treatment regimen involving norfloxacin, nalidixic acid, co-trimoxazole tetracycline and ampicillin are specific for *Vibrio* mediated diarrhea (24). Other antibiotics were also screened in the study to investigate the common resistance profiles of *Vibrio spp.* Accordingly Kirby Bauer's disk diffusion test was performed against fifteen different antibiotics (Figure 10 a-d).

In β–lactam class, alarming resistance levels were analyzed for ceftazidime (65.5%), cefipime (62%) and ceftriaxone (55.1%) antibiotics (Figure 10a). The proportion of intermediate isolates was found from 6.8% to 20.6% for all β–lactam antibiotics. However, lowest resistance was observed for imipenem (13.8%). Therefore, elucidating imipenem as most effective β–lactam antibiotic against *Vibrio* infections.

Among aminoglycosides, highest frequency of resistance was observed against streptomycin (34.4%) and kanamycin (31%) (Figure 10b). Percentage of intermediate isolates for

aminoglycosides group ranged from 6.8% to 31%. *Vibrio spp.* exhibited minimum resistance levels for netilimicin (6.8%) followed by tobramycin (13.7%) and amikacin (20.6%).

For quinolones, alarming resistance rates were observed for nalidixic acid (72.4%) followed by ofloxacin (37.9%) and ciprofloxacin (37.9%), and norfloxacin (27.4%) (Figure 10c). *Vibrio spp.* exhibited least resistance against levofloxacin (30.1%). Proportions of intermediate isolates for quinolones were observed to range between 3.4% - 20.6%.

Overall, *Vibrio* isolates were most sensitive for imipenem, netilmicin, tobramycin, levofloxacin and norfloxacin hence, these antibiotics could be priority antibiotics of choice for treatment of *Vibrio* infections. Moreover, continuous monitoring of antibiotic resistance is required to access β-lactam resistance trends as third generation cephalosporin resistant *Vibrio* incidences are rare and anxiously alarming (Li *et al* ., 2015; WHO, 2005).

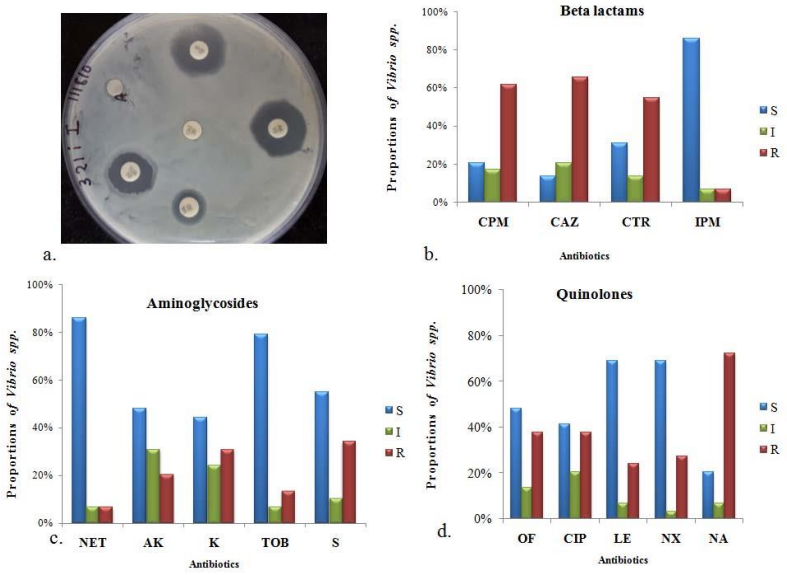


Figure 10 Antibiotic susceptibility patterns of *Vibrio spp.* a: Antibiotic susceptibility performed by Kirby Bauer's disk diffusion method. Proportions of sensitive (S), intermediate (I) and resistant (R) phenotypes for β lactams (b), aminoglycosides (c) and quinolones (d). CPM- Cefepime, CAZ- Ceftazidime, CTR- Ceftriaxone, IPM- Imipenem, NET- Netilmicin, AK- Amikacin, K- Kanamycin, TOB- Tobramycin, S- Streptomycin, OF- Ofloxacin, CIP- Ciprofloxacin, LE- Levofloxacin, NX- Norfloxacin, NA- Nalidixic acid.

Discussion:

Successful interventions in management of infectious diseases need identification of the etiological agent and treatment of clinical symptoms manifested during disease. Diarrhea is a multifactorial illness associated with wide spectrum of pathogens including viruses, bacteria and parasites (Liu *et al.,* 2012; Saeed *et al.,* 2015). Conventional microbiological techniques combined with molecular identification system incredibly increases reproducibility and scalability of etiological agent characterization. However, in context of diarrhea, the viral agents have been explored to a greater extent as compared to others (Badur *et al.,* 2015 Kang *et al.,* 2016; Sarkar *et al.,* 2016).

Present study elucidated incidences of DEC, *Vibrio spp., Salmonella spp.* and *Shigella spp.* among diarrheal patients. However, owing to higher incidences of DEC and *Vibrio spp.,* bacteria were characterized using 16S rRNA gene amplification. DEC were further characterized at molecular and serological levels to identify different pathotypes and serogroups. The incidence rates of diarrheagenic *E. coli* were observed up to 21% as the sole pathogen & approximately 6% as mixed infection with Rota virus. Our study shows moderate DEC infection rates, similar to the reports from developing world (Paredes-Paredes *et al.,* 2011; Alikhani *et al.,* 2013; Heidary *et al.,* 2014; Canizalez-Roman *et al.,* 2016). However reports from other parts of India and neighboring countries showed 10-35% variation in DEC incidence rates (Shetty *et al .,* 2012; Suganya *et al.,* 2016; Zhu *et al.,* 2016; Younas *et al.,* 2016). Sporadic outbreaks with 42% to 65% of incidences are also reported from different regions of India (Rajendran *et al.,* 2010; Sudershan *et al .,* 2014). Globally, prevalence of *E. coli* as an etiological agent of diarrhea is well reported between 30%- 40% cases (Behiry *et al.,* 2012; Acosta *et al.,* 2016). DEC co-infection with other enteric pathogens is greatly known to aggravate symptoms and duration of diarrhea (Tobias *et al.,* 2015; Zhu *et al.,* 2016).

High frequencies of the *eae* gene in current study underpin the importance of atypical EPEC as predominant diarrheal pathogen in the region. Low frequency of *bfpA* observed in the present study suggests its fewer incidence rates in the population similar to the previous reports (Cabal *et al.,* 2016; Dias *et al.,* 2016). Among the ETEC positive patients, *estA* gene was more frequently isolated than *eltB* alone or *estA* and *eltB* in combination is similar to other studies (Dutta *et al.,* 2012; Nguyen *et al.,* 2005). For many years ETEC has been implicated as the major cause of traveler's diarrhea (Vilchez *et al.,* 2012). In the present study ETEC showed varying prevalence

among all ages and similar observations were reported from the northern part of the country (Chandra *et al.,* 2012; Rajeshwari *et al.,* 2015). We observed EAEC predominantly in children (n=7/8) followed by elderly age group (1/8). Other studies have also shown prevalence of *pCVD* positive *E. coli* in the stool specimens of adults and childhood diarrhea and this can be as high as 11% (Aslani *et al.,* 2011). Current findings strengthen evidences that EAEC is an emerging diarrheal agent in the South East Asian children population (Pablan *et al.,* 2013).

As different DEC molecular pathotypes exhibit surface to invasive pathophysiology resulting in different clinical outcomes. We found that clinical symptoms of watery stools and mucus were significantly associated with DEC pathotype infection. Present observations reinforce the conviction that DEC pathotype are considerably responsible for severe gastrointestinal infections associated with childhood and adult diarrhea (Saeed *et al.,* 2015).

In past few years, increased incidences of *Vibrio* have been reported from various parts of the globe (WHO, 2017) with coastal regions more prone to this infectious disease (Taneja *et al.,* 2012; WHO, 2017). Historically, *Vibrio* has caused many pandemic and epidemics along with several explosive outbreaks which continue to thrive in regions with contaminated water and suboptimal hygiene practices (CDC, 2017; Mukhopadhyay *et al*. 2014; Pal *et al.,* 2017).

By amplification of *Vibrio* genus specific 16S rRNA gene, infection rate of *Vibrio* was found to be 5 % (29/572) among diarrheal population (Figure 6). In a previous study, Bora & coworkers reported 16.5% of *Vibrio* infections in an outbreak from remote areas of Himachal Pradesh (Bora *et al.,* 1997). In addition, such higher *Vibrio spp.* incidences have also been reported from surrounding areas of state (Jaiswal *et al.,* 2015; Mutreja *et al.,* 2011). Another study deciphered presence of higher proportion of *Vibrio spp.* in water samples collected from different river systems (Kumar *et al.,* 2014). Similarly, *Vibrio* incidence rates of 2% to 4% have also been reported from surroundings of Himachal Pradesh (Mala *et al.,* 2014; Kumar *et al.,* 2014; Uppal *et al .,* 2016). In comparison to our observation, higher *Vibrio* infection rates ranging from 10% - 15% are reported from different parts of India (Dutta *et al.,* 2013; Chhotray *et al.,* 2017). Furthermore, surveillance studies from Bangladesh reported up to 18% of *Vibrio* infections among diarrheal populations (Paul *et al.,* 2016; Rashed *et al.,* 2017). Overall, our study depicts low levels of *Vibrio spp.* incidences and similar trends are observed for active surveillance studies in the country.

Our observations indicate higher proportions of DEC pathotypes associated with childhood diarrhea than any other age set. In a recent study conducted in Mexico, Canizalez-Roman and coworkers also reported higher DEC incidences in children population (Canizalez-Roman *et al.*, 2016). In addition, higher frequencies of DEC pathotypes in moderate to severe cases of childhood diarrhea are reported all over the globe (Kotloff *et al.*, 2012; Dutta *et al .*, 2013; Tobias *et al.*, 2015). Ambiguously, incidences of *Vibrio* were found to be higher among adolescent and adult population and similar observations were found in other report (Shreshtha *et al.*, 2015). However, children less than five years age group (0-2 and 3-5 years) also suffered with similar infection rates as that of adult. CDC weekly morbidity and mortality reports also found children less than five years as most vulnerable group to *Vibrio* infection (Crim *et al.*, 2015).

Season wise distribution analysis of DEC showed prevalence throughout the years. Seasonal variations for *Vibrio* incidences are driven by various factors like recurrent floods stagnant water and suboptimal hygienic conditions (Ramamurthy *et al.*, 2003; Mookerjee *et al.*, 2014). Annually, Himachal Pradesh is fed by snow and rainfall however, owing to its mountainous slopes water is drained into two main basins; Indus and Gangetic river systems. Uniform seasonal distribution of *Vibrio spp.* were seen throughout the years however slight peaks were observed during rainy, winter and summer seasons. Similar seasonal trends have been observed from coastal area but frequency is much higher as compared to current study (Pal *et al.*, 2017; Rashed *et al.*, 2017; Shreshtha *et al.*, 2015; Ramamurthy *et al.*, 2003; Mookerjee *et al.*, 2014; Deepanjali *et al.*, 2005). This may be due to lack of stagnant water reservoirs in the region and direct drainage of rain water into major river systems.

In the present study EPEC was found as most the resistant pathotype and highest levels of antibiotic resistance were observed against ampicillin. These observations are concordance with previous studies analyzing DEC resistance (Darehabi *et al.*, 2013; Amaya *et al.*, 2014). We observed lowest resistance rates against co- trimoxazole among all DEC pathotypes. However, Sadeghabadi and coworkers reported approximately 80% resistance against co- trimoxazole in diarrheagenic *E. coli*. Similar reports across the globe also elucidated high levels of resistance against DEC pathotypes (Rajeshwari *et al.*, 2015; Sadeghabadi *et al.*, 2014; Haghi *et al.*, 2014).

in addition, Vibrio strains showed greatest sensitivity levels against imipenem (86.2%) followed by netillin, tobramycin, levofloxacin, ofloxacin, amikacin and kanamycin (Figure 10a to 10d).

Similar findings have also been reported from other parts of country (Pal *et al.,* 2017; Mala *et al.,* 2014; Kumar *et al.,* 2014; Uppal *et al.,* 2017)**.** Moreover our study showed current treatment regimen effective against *Vibrio* isolates.

Moreover, *Vibrio* strains showed rising levels of resistance against beta lactam group (cefepime, ceftazidime and ceftriaxone) and nalidixic acid. *Vibrio* strains having resistance against cephalosporins have been reported scarcely (Mandal *et al.,* 2012) while, the current study elicited alarming resistance rates against cephalosporin class in the region. Previous studies reported very higher levels of resistance against nalidixic acid, streptomycin and ciprofloxacin antibiotics (Mala *et al.,* 2014; Kumar *et al.,* 2014; Garg *et al.,* 2000; Ghosh *et al* ., 2016) however our study implicated relatively low levels of resistance against these antibiotics.

Summary & conclusion:

Present study is to our knowledge, the first comprehensive research in the region addressing associations of bacterial pathogens with clinical outcomes and antibiogram patterns. Our findings highlight the importance of continuous DEC pathotype surveillance programs for therapeutic approaches and not the least the benefit of employing comprehensive inspection of antimicrobial resistance in the region. In relation to treatment, a very few studies have evaluated comprehensive importance of drugs for the management of DEC pathotype infection. After introduction of the rotavirus vaccines into national immunization program of India, the next priority must be to identify diarrheal pathogens owing to high morbidity and mortality rates. The study would help in prioritizing diagnostic and therapeutic measures against predominant DEC pathotypes. Exploring the resistant phenotypes would aid in management & spread of multidrug resistant strains. Following are highlights from the study;

- A total of 572 stool samples were screened for characterization of bacterial pathogens.
- Diarrheagenic *E. coli* was characterized as predominant pathogen in 21% samples followed by *Vibrio spp.* (4.7%), *Salmonella spp.* (0.8%), and *Shigella spp.* (0.3%).
- EPEC was detected as predominant *E. coli* pathotypes (13.7%), followed by ETEC (5.7%) and EAEC (1.3%), pathotypes.
- DEC molecular pathotypes infection rates were higher among children under the age of five, while *Vibrio spp.* infection rates were high among adolescent and adult age groups.

- Serological characterization of DEC elucidated 23 serogroups consisting of O26, O2 and O41 as predominant one.
- Incidences of DEC and *Vibrio spp.* infections were observed throughout the year however infection peaks were seen during summer and rainy seasons.
- Alarming rates of resistance were seen against commonly used antibiotic regime.

References:

1. G.J. Acosta, N.I. Vigo, D. Durand *et al.* Diarrheagenic *Escherichia coli*: Prevalence and pathotype distribution in children from Peruvian rural communities. The American Journal of Tropical Medicine and Hygiene. Vol 95, pp 574-579, 2016.
2. Y. Alikhani, S. Mohammad. Prevalence and antibiotic resistance patterns of diarrheagenic *Escherichia coli* isolated from adolescents and adults in Hamedan, Western IranIranian Journal of Microbiology. Vol 5, pp 42-47, 2013.
3. S. Almagro-Moreno, K. Pruss and R. Taylor. Intestinal Colonization Dynamics of *Vibrio cholera*. PLoS pathogens,2015;11:e1004787.
4. E. Amaya, S. Vilchez, M. Paniagua, R. Möllby, C. Nord, A. Weintraub and D. Reyes. Antibiotic resistance patterns of intestinal *Escherichia coli* isolates from Nicaraguan children. Journal of Medical Microbiology. Vol 60, pp. 216-222, 2011.
5. M.M. Aslani. Characterization of enteroaggregative *Escherichia coli* (EAEC) clinical isolates and their antibiotic resistance pattern. The International Journal of Infectious Diseases. 2011;15:e136-e139.
6. M. Badur, N.M. Latha, P.R. Kumar *et al* . Prevalence of Rotavirus diarrhea among under-5 hospitalized children in a Government tertiary hospital, Tirupati. Journal Dr.NTR university of Health sciences. Vol 4, pp. 112, 2015.
7. A.W. Bauer, D.M. Perry and W.M. Kirby. Single-disk antibiotic-sensitivity testing of staphylococci: An analysis of technique and results. A.M.A. archives of internal medicine American Medical Association. Vol 104, pp.208-216, 1959.
8. I. Behiry, E. Abada, E. Ahmed and R. Labeeb. Enteropathogenic*Escherichia coli*Associated with Diarrhea in Children in Cairo, Egypt. The Scientific World Journal. Vol 11, pp. 2613-2619, 2012

9. D. Bora, A. Dhariwal, D. Jain, V. Sachdeva, J. Vohra, R. Prakash, K. Datta and R. Sharma. *V. cholerae* 01 outbreak in remote villages of Shimla district, Himachal Pradesh, 1994. The Journal of Communicable Diseases. Vol 29 Issue 2. pp. 121-5, June 1997.
10. A. Cabal, M. García-Castillo, R. Cantón, C. Gortázar, L. Domínguez and J. Álvarez. Prevalence of *Escherichia coli* Virulence Genes in Patients with Diarrhea and a Subpopulation of Healthy Volunteers in Madrid, Spain. Frontiers in Microbiology. 2016; Vol 7, pp. 641, May 2016.
11. L. Campos, M. Franzolin and L. Trabulsi. Diarrheagenic *Escherichia coli* categories among the traditional enteropathogenic *E. coli* O serogroups: a review. Memorias do Instituto Oswaldo Cruz. Vol 99 Issue 6, pp. 545-552, October 2004.
12. A. Canizalez-Roman, H. Flores-Villaseñor, E. Gonzalez-Nuñez, J. Velazquez-Roman, J. Vidal, S. Muro-Amador, G. Alapizco-Castro, J. Díaz-Quiñonez and N. León-Sicairos. Surveillance of Diarrheagenic *Escherichia coli* Strains Isolated from Diarrhea Cases from Children, Adults and Elderly at Northwest of Mexico. Frontiers in Microbiology. Vol 7, pp. 1924, November 2016.
13. B. Chandra, G. Singh, N. Taneja, S. Pahil, S. Singhi and M. Sharma. Diarrhoeagenic *Escherichia coli* as a predominant cause of paediatric nosocomial diarrhoea in India. Journal of Medical Microbiology. Vol 61, pp. 830-836, June 2012.
14. The Pretravel Consultation - Chapter 2. Yellow Book | Travelers' Health | CDC", Wwwnc.cdc.gov, Available: https://wwwnc.cdc.gov/travel/yellowbook/2018/the-pre-travel-consultation/the-pre-travel-consultation
15. G.P. Chhotray, M.R. Ranjit, B.B. Pal, P.K. Meher and H.K. Khuntia. Incidence and Molecular Analysis of *Vibrio cholerae* among Some Primitive Tribes in Odisha, India. International Journal of Current Microbiology and Applied Sciences. Vol 6, pp. 51-61, 2017.
16. S. Crim, P. Griffin, R. Tauxe, E. Marder, D. Gilliss, A. Cronquist, M. Cartter, M. Tobin-D. Angelo, D. Blythe, K. Smith, S. Lathrop, S. Zansky, P. Cieslak, J. Dunn, K. Holt, B. Wolpert and O. Henao. Preliminary incidence and trends of infection with pathogens transmitted commonly through food - Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2014. Morbidity and mortality weekly report Centers for Disease Control and Prevention. Vol 64 Issues 18, pp. 495-499, May 2015.

17. H.K. Darehabi, M.H. Naseri, S. Menbari, J. Mobaleghi, E. Kalantar. Antibiotic resistance pattern of *Escherichia coli* groups A, B1, B2 and D isolated from frozen foods and children with diarrhea in Sanandaj. International Journal of Enteric Pathogens. Vol 1 Issues 1, pp. 1-4, August 2013.
18. C. Debroy, B. Brigh, R. Wilson, J. YeaL, R. Kumar and M. Bhan. Plasmid-coded DNA fragment developed as a specific gene probe for the identification of enteroaggregative *Escherichia coli*. Journal of Medical Microbiology. Vol 41 issue 6, pp. 393-398, 1994.
19. A. Deepanjali, H. Kumar, I. Karunasagar and I. Karunasagar. Seasonal Variation in Abundance of Total and Pathogenic *Vibrio parahaemolyticus* Bacteria in Oysters along the Southwest Coast of India. Applied and Environmental Microbiology. Vol 71 issue 7, pp. 3575-80, July 2005.
20. R. Dias, B. dos Santos, L. dos Santos, M. Vieira, R. Yamatogi, A. Mondelli, T. Sadatsune, J. Sforcin, T. Gomes and R. Hernandes. Diarrheagenic *Escherichia coli*pathotypes investigation revealed atypical Enteropathogenic *E. coli* as putative emerging diarrheal agents in children living in Botucatu, São Paulo State, Brazil", Acta pathologica, microbiologica, et immunologica Scandinavica (APMIS). Vol 124 Issue 4, pp. 299-308, April 2016.
21. A. Dupont, F. Sommer, K. Zhang, U. Repnik, M. Basic, A. Bleich, M. Kühnel, F. Bäckhed, Y. Litvak, M. Fulde, I. Rosenshine and M. Hornef. Age-Dependent Susceptibility to Enteropathogenic *Escherichia coli* (EPEC) Infection in Mice. PLoS Pathogens. May 2016;12:e1005616.
22. D. Dutta, G. Chowdhury, G. Pazhani, S. Guin, S. Dutta, S. Ghosh, K. Rajendran, R. Nandy, A. Mukhopadhyay, M. Bhattacharya, U. Mitra, Y. Takeda, G. Nair and T. Ramamurthy. *Vibrio cholera*eNon-O1, Non-O139 Serogroups and Cholera-like Diarrhea, Kolkata, India. Emerging Infectious Diseases. Vol 19 Issue 3, pp. 464-467, March 2013.
23. S. Dutta, S. Guin, S. Ghosh, *et al* . Trends in the prevalence of diarrheagenic *Escherichia coli* among hospitalized diarrheal patients in Kolkata, India. PloS one. February 2013 8:e56068.
24. W. Elias and F. Navarro-Garcia. Enteroaggregative *Escherichia coli* (EAEC). *Escherichia coli* in the Americas. Springer. pp. 27-57, October 2016.

25. P. Feng, S.D. Weagant, K. Jinneman. BAM: diarrheagenic *Escherichia coli*. 2014. Available:http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070080.html
26. C. Frank, D. Werber, J.P. Cramer, *et al.*, Epidemic profile of Shiga-toxin–producing *Escherichia coli* O104: H4 outbreak in Germany. The New England Journal of Medicine 2011;365:1771-1780.
27. P. Garg, S. Chakraborty, I. Basu, S. Datta, K. Rajendran, T. Bhattacharya, S. Yamasaki, S. Bhattacharya, Y. Takeda, G. Nair and T. Ramamurthy, "Expanding multiple antibiotic resistance among clinical strains of *Vibrio cholerae* isolated from 1992–7 in Calcutta, India" Epidemiol & Infect 2000;124(3):393-399.
28. R. Ghosh, N. Sharma, K. Halder, R. Bhadra, G. Chowdhury, G. Pazhani, S. Shinoda, A. Mukhopadhyay, G. Nair and T. Ramamurthy, "Phenotypic and Genetic Heterogeneity in *Vibrio cholera*e O139 Isolated from Cholera Cases in Delhi, India during 2001–2006", Frontiers in Microbiology2016;7.
29. R. Guzman-Hernandez, A. Contreras-Rodriguez, R. Hernandez-Velez, I. Perez-Martinez, A. Lopez-Merino, M. Zaidi and T. Estrada-Garcia, "Mexican unpasteurised fresh cheeses are contaminated with *Salmonella spp*., non-O157 Shiga toxin producing *Escherichia coli* and potential uropathogenic *E. coli* strains: A public health risk", Int J Food Microbiol. 2016;237:10-16.
30. F. Haghi, H. Khoshvaght, H. Zeighami, M. Bayat and F. Hajiahmadi, "Frequency and antimicrobial resistance of diarrhoeagenic *Escherichia coli* from young children in Iran", Journal of Medical Microbiology. 2014;63:427-432.
31. M. Heidary, H. Momtaz and M. Madani, "Characterization of Diarrheagenic Antimicrobial Resistant *Escherichia coli* Isolated From Pediatric Patients in Tehran, Iran", Iranian Red Crescent Medical Journal. 2014;16.
32. https://www.cdc.gov/cholera/asia/index.html#one last. Last accessed on 2017 Sept10].
33. A. Jaiswal, S. Sarkar, P. Das, S. Nandy, H. Koley and B. Sarkar, "Trends in the genomic epidemiology of *Vibrio cholerae* O1 isolated worldwide since 1961", Int J Antimicrob Agents 2015;46:460-464.
34. Jensen BH, Olsen KE, Struve C, *et al* . Epidemiology and clinical manifestations of enteroaggregative *Escherichia coli*. Clin Microbiol Rev. 2014;27:614-630.

35. B. Hebbelstrup Jensen, K. Olsen, C. Struve, K. Krogfelt and A. Petersen, "Epidemiology and Clinical Manifestations of Enteroaggregative *Escherichia coli*", App Environ Microbiol 2003;69:7541-7544.
36. G. Kang, "Rotavirus in India: Forty years of research", 2016;53:569-573.
37. J.B. Kaper, J.G. Morris, M.M. Levine, Cholera. Clin Microbiol Rev 1998;8:48-86.
38. J. Kaper, J. Nataro and H. Mobley, "Pathogenic *Escherichia coli*", Nature Rev Microbiol. 2004;2:123-140.
39. K. Kotloff, W. Blackwelder, D. Nasrin, J. Nataro, T. Farag, A. van Eijk, R. Adegbola, P. Alonso, R. Breiman, A. Golam Faruque, D. Saha, S. Sow, D. Sur, A. Zaidi, K. Biswas, S. Panchalingam, J. Clemens, D. Cohen, R. Glass, E. Mintz, H. Sommerfelt and M. Levine, "The Global Enteric Multicenter Study (GEMS) of Diarrheal Disease in Infants and Young Children in Developing Countries: Epidemiologic and Clinical Methods of the Case/Control Study", 2012;55:S232-S245.
40. A. Oberoi and A. Kumar, "*Vibrio* isolates from cases of acute diarrhea and their antibiotic susceptibility pattern in a tertiary care hospital of Punjab", CHRISMED J Health & Res 2014; 1:254.
41. X. Li, D. Wang, B. Li, H. Zhou, S. Liang, C. Ke, X. Deng, B. Kan, J. Glenn Morris, W. Cao, "Characterization of environmental *Vibrio cholerae* serogroups O1 and O139 in the Pearl River Estuary, China" Canadian J Microbiol 2015;62:139-147.
42. E. Lipp, I. Rivera, A. Gil, E. Espeland, N. Choopun, V. Louis, E. Russek-Cohen, A. Huq and R. Colwell, "Direct Detection of *Vibrio cholera*e and *ctxA* in Peruvian Coastal Water and Plankton by PCR", App Environ Microbiol 2003;69(6):3676-3680.
43. L. Liu, H. Johnson, S. Cousens, J. Perin, S. Scott, J. Lawn, I. Rudan, H. Campbell, R. Cibulskis, M. Li, C. Mathers and R. Black, "Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000", The Lancet. 2012;379:2151-2161.
44. E. Mala, A. Oberoi, V.S. Alexander, "*Vibrio* isolates from cases of acute diarrhea and their antimicrobial susceptibility pattern in a tertiary care hospital" Int J Basic & Appl Sci 2014;3:35-37.

45. J. Mandal, K.P. Dinoop, S.C. Parija, “Increasing Antimicrobial Resistance of *Vibrio cholerae* OI Biotype EI Tor Strains Isolated in a Tertiary-care Centre in India” J health Popul Nutr 2012;30(1):12-16.
46. E. Miliwebsky, F. Schelotto, G. Varela, D. Luz, I. Chinen and R. Piazza, "Human Diarrheal Infections: Diagnosis of Diarrheagenic *Escherichia coli* Pathotypes", *Escherichia coli* in the Americas 2016;343-369).
47. S. Mookerjee, A. Jaiswal, P. Batabyal, M. Einsporn, R. Lara, B. Sarkar, S. Neogi and A. Palit, "Seasonal dynamics of *Vibrio cholera*e and its phages in riverine ecosystem of Gangetic West Bengal: cholera paradigm", Environ Monitor and Assess 2014;186:6241-6250.
48. A. Mukhopadhyay, Y. Takeda and G. Balakrish Nair, "Cholera Outbreaks in the El Tor Biotype Era and the Impact of the New El Tor Variants,” Curr Top Microbiol Immunol 2014;379:17-47
49. A. Mutreja, D.W. Kim, N. Thomson, T.R. Connor, J.H. Lee, *et al*. Evidence for multiple waves of global transmission within the seventh cholera pandemic. Nature, 477:462, 2011.
50. G.B. Nair, T. Ramamurthy, S.K. Bhattacharya, A.K. Mukhopadhyay, S. Garg, M.K. Bhattacharya, *et al*. Spread of *Vibrio cholerae* 0139 Bengal in India. Journal of Infect Dis, Vol 169, pp. 1029-1034, 1994.
51. J.P. Nataro, T.B. Kaper. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. Vol 11 pp. 142-201, 1998.
52. T.V. Nguyen, P. Le Van, C. Le Huy, *et al.* Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam. J Clin Microbiol. Vol 43 pp. 755-760, 2005.
53. M. O’Ryan, V. Prado, L.K. Pickering. A millennium update on pediatric diarrheal illness in the developing world, pp. 125-136. *In* (ed), Elsevier.
54. S. Ochi, M. Shah, E. Odoyo, *et al.* An Outbreak of Diarrhea in Mandera, Kenya, Due to *Escherichia coli* Serogroup O-Nontypable Strain That Had a Coding Gene for Enteroaggregative *E. coli* Heat-Stable Enterotoxin 1.ASTMH, 16-0310, 2016.
55. P.C. Okhuysen, H.L. DuPont. Enteroaggregative *Escherichia coli* (EAEC): a cause of acute and persistent diarrhea of worldwide importance. JID. Vol 202, pp. 503-505, 2010.

56. F. Ørskov, I. Ørskov. "Serotyping of *Escherichia coli*," in Methods in Microbiology Vol. 14 ed. Bergan T., editor. (London: Academic Press) 43–112, 1984.
57. M. Paredes-Paredes, P.C. Okhuysen, J. Flores, et al. Seasonality of diarrheagenic *Escherichia coli* pathotypes in the US students acquiring diarrhea in Mexico. Journal of Travel Medicine. Vol 18, pp.121-125, 2011.
58. J.B. Patel, F.R. Cockerill, P.A. Bradford, *et al*. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. 2015;3(1):1-240.
59. R.C. Paul, A.S. Faruque, M. Alam, A. Iqbal, K. Zaman, N. Islam, A. Sobhan, S.K. Das, M.A. Malek, F. Qadri, A. Cravioto. Incidence of severe diarrhea due to *Vibrio cholerae* in the catchment area of six surveillance hospitals in Bangladesh. Epidemiology and Infection. Vol 144, pp. 927-939, 2016.
60. V. Radosavljevic, E.J. Finke and G. Belojevic. Analysis of *Escherichia coli* O104: h4 Outbreak in Germany in 2011 Using Differentiation Method for Unusual Epidemiological Events. Central European Journal of Public Health. Vol 24 issue 1, pp. 9-15, 2016.
61. P. Rajendran, S.R. Ajjampur, D. Chidambaram. *et al.* Pathotypes of diarrheagenic *Escherichia coli* in children attending a tertiary care hospital in South India. Diagnostic Microbiology and Infectious Disease. Vol 68, pp. 117-122, 2010.
62. K. Rajeshwari, B. Uppal, R. Singh, *et al*. Multidrug resistant enteropathogenic *E. coli* diarrhea in children. American Journal of Research Communication. Vol 3, pp. 27-48, 2015.
63. T. Ramamurthy, S. Yamasaki, Y. Takeda, G.B. Nair. *Vibrio cholerae* O139 Bengal: odyssey of a fortuitous variant. Microbes and Infection. Vol 5, pp. 329–344, 2003.
64. S.M. Rashed, N.A. Hasan, M. Alam, A. Sadique, M. Sultana, M.M. Hoq, *et al. Vibrio cholerae* O1 with reduced susceptibility to ciprofloxacin and azithromycin isolated from a rural coastal area of Bangladesh. Frontiers in Microbiology. pp. 8, 2017.
65. D.A. Rasko, D.R. Webster, J.W. Sahl, *et al*. Origins of the *E. coli* strain causing an outbreak of hemolytic–uremic syndrome in Germany. The New England Journal of Medicine. Vol 365, pp. 709-717, 2011.

66. G. Sabat, P. Rose, W.J. Hickey and J.M. Harkin. Selective and sensitive method for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. Applied and Environmental Microbiology. Vol 66 Issue 2, pp. 844-849, 2000.
67. A.F. Sadeghabadi, A. Ajami, R. Fadaei, *et al*. Widespread antibiotic resistance of diarrheagenic *Escherichia coli* and *Shigella* species. Journal of Research in Medical Sciences. Vol 19:S51, 2014.
68. A. Saeed, H. Abd and G. Sandstrom. Microbial aetiology of acute diarrhoea in children under five years of age in Khartoum, Sudan. Journal of Medical Microbiology. 2015; 64:432-437.
69. R. Sarkar, B. Gladstone, J. Warier *et al*. Rotavirus and other diarrheal disease in a birth cohort from Southern Indian community. Indian Pediatrics. Vol 53, pp. 583-588, 2016.
70. V.A. Shetty, S.H. Kumar, A.K. Shetty *et al*. Prevalence and characterization of diarrheagenic *Escherichia coli* isolated from adults and children in Mangalore, India. Journal of Laboratory Physicians. Vol 4, pp. 24, 2012.
71. U.T. Shrestha, N. Adhikari, R. Maharjan, M.R. Banjara, K.R. Rijal, S.R. Basnyat, *et al*. Multidrug resistant *Vibrio cholerae* O1 from clinical and environmental samples in Kathmandu city. BMC Infectious Diseases. Vol 15, pp. 104, 2015.
72. Standard operative procedures bacteriology. 2015. Available from: www.icmr.nic.in/Publications/SOP/SOP_Bacteriology.pdf
73. R. Sudershan, R.N. Kumar, B. Kulkarni, *et al*. *E. coli* pathotypes and their antibiotic resistance in young children with diarrhea in Hyderabad, India. International Journal of Current Microbiology and Applied Sciences. Vol 3, pp. 647-654, 2014.
74. D. Suganya, K. Kanimozhi, A. Panneerselvam, *et al*. Molecular Characterization of Diarrheagenic *Escherichia coli* from Tiruchirappalli District, Tamilnadu, India. International Journal of Current Microbiology and Applied Sciences. Vol 5, pp. 478-484, 2016.
75. M. Tahden, J. Manitz, K. Baumgardt, *et al*. Epidemiological and Ecological Characterization of the EHEC O104: H4 Outbreak in Hamburg, Germany, 2011. PloS one. 11:e0164508, 2016.
76. N. Taneja, G. Sangar, G. Chowdhury, T. Ramamurthy, A. Mishra, M. Singh, *et al*. Molecular epidemiology of *Vibrio cholerae* causing outbreaks & sporadic cholera in

northern India. The Indian Journal of Medical Research. Vol 136, Issue 4, pp. 656-663, 2012.

77. J. Tobias, E. Kassem, U. Rubinstein, *et al*. Involvement of main diarrheagenic *Escherichia coli*, with emphasis on enteroaggregative *E. coli*, in severe non-epidemic pediatric diarrhea in a high-income country. BMC Infectious Diseases. Vol 15 pp. 79, 2015.
78. B. Uppal, B. Mehra, P.S. Panda and S.K. Kumar. Antimicrobial susceptibility profile of *Vibrio cholerae* strains isolated at a tertiary care medical centre in New Delhi, India. The International Journal of Community Medicine and Public Health. Vol 4, pp. 868-871, 2017.
79. S. Vilchez, S. Becker-Dreps, E. Amaya, C. Perez, M. Paniagua, D. Reyes, F. Espinoza, A. Weintraub. Characterization of enterotoxigenic *Escherichia coli* strains isolated from Nicaraguan children in hospital, primary care and community settings. Journal of Medical Microbiology. Vol 63, pp. 729-734, 2012.
80. A. Weintraub. Enteroaggregative *Escherichia coli*: epidemiology, virulence and detection. Journal of Medical Microbiology. Vol 56, pp. 4-8, 2007.
81. World Health Organization, UNICEF. Clinical management of acute diarrhea: WHO.
82. World Health Organization. Global Health Observatory (GHO): Number of reported cholera cases. http://www.who.int/gho/epidemic_diseases/cholera/cases_text/en/. [Last accessed on 2017 Sept10].
83. M. Younas, F. Siddiqui, Z. Noreen, *et al*. Characterization of enteropathogenic *Escherichia coli* of clinical origin from the pediatric population in Pakistan. Trans R Soc Trop Med Hyg. Vol 110 pp. 414-420, 2016.
84. H. ZX, Hu, L. Tian, Z.J. Cheng, *et al*. Viral and Bacterial Etiology of Acute Diarrhea among Children under 5 Years of Age in Wuhan, China. Chinese Medical Journal. Vol 129 pp.1939, 2016.
85. N. Pabalan, E. Singian, H. Jarjanazi, *et al*. Enteroaggregative *Escherichia coli* and acute diarrhea in children: a meta-analysis of South Asian populations. European Journal of Clinical Microbiology & Infectious Diseases. Vol 32, pp. 597-607, 2013.

86. B.B. Pal, H.K. Khuntia, S.P. Nayak, A. Mohanty, B. Biswal. *Vibrio cholerae* O1 Ogawa strains carrying the ctxB7 allele caused large cholera outbreak during 2014 in the tribal areas of Odisha, India. Japanese Journal of Infectious Diseases. 2017:JJID-2016.
87. S. Panchalingam, M. Antonio, A. Hossain, *et al*. Diagnostic microbiologic methods in the GEMS-1 case/control study. CID. 2012;55:S294-S302.